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Major Antifungals in Nutmeg Essential Oil against *Aspergillus flavus* and *A. ochraceus*

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Abstract

Aiming to substitute toxic synthetic fungicides, the activity of nutmeg (*Myristica fragrans*) essential oil (EO, obtained by hydrodistillation) was investigated against two important storage fungi-*Aspergillus flavus A. ochraceus*. The activity of crude nutmeg EO was investigated using poison food assay (PFA). At a concentration of 0.1%, the EO inhibited *A. flavus* and *A. ochraceus* growth by 43 and 65%, respectively. At a concentration of 0.3 %, *A. flavus* and *A. ochraceus* inhibitions were 84 and 79%, respectively. The crude nutmeg EO on fractionation by preparative TLC-bioautography presented one band from which two pure compounds were isolated by semi-preparative normal-phase high performance liquid chromatography. Myristicin and safrole were identified by nuclear magnetic resonance (^{1}H and ^{13}C) and gas chromatography-mass spectrometry. The relative % of myristicin and safrol in the crude EO was 10.8 and 2.9, respectively, determined by gas chromatography with a flame ionization detector. The crude EO, the isolated active fraction, isolated myristicin and standard myristicin presented similar activities against the two fungi at concentrations of 0.1 and 0.3% by PFA. Based on these results it is concluded that myristicin is the major antifungal in nutmeg EO against *A. flavus* and *A. ochraceus*.

Keywords: *Aspergillus flavus*, *Aspergillus ochraceus*, essential oil, *Myristica fragrans,* mycotoxigenic fungi, nutmeg

1. Introduction

The fungi *Aspergillus. flavus* and *A. ochraceus* cause post-harvest deterioration of fruit, vegetables and grains in tropical ecosystems such as Brazil (Rodríguez-Amaya & Sabino, 2002; Kumar et al., 2008). Synthetic fungicides used to control/manage postharvest fungi present several limitations, such as, carcinogenicity, teratogenicity, high toxicity, long degradation periods, environmental pollution and negative effects in humans. Thus, naturally occurring antimicrobials in essential oils (EOs) have been receiving increasing attention (Jardim et al., 2008, 2010; Reddy et al., 2009; Valente et al., 2011).

Nutmeg is the seed of *Myristica fragrans* Houtt (Myristicaceae), a medium-size evergreen tree native to the Moluccas or Spice Island of Indonesia, cultivated in the tropics, with Indonesia and Sri Lanka being the major producers. More recently, its production in northeastern region (Bahia) of Brazil has increased significantly. Nutmeg has been extensively used as a spice and flavouring agent in the food industry and domestic use since ages It presents insecticidal (Huang et al., 1997) and anti-oxidant (Olaleye et al., 2006) properties.

Antifungal properties of nutmeg EO have been extensively studied (Chatterjee, 1990; Feng & Zheng, 2007; Kamble & Patil, 2008; Valente et al., 2011). While the EO chemical compositions have been extensively studied over the last century (Sanford & Heinz, 1971; Forrest & Heacock, 1972; Schenk & Lamparsky, 1981; Choo et al., 1999; Pino & Borges, 1999; Spricigo et al., 1999; Wang et al., 2004; Jukić et al., 2006; Yuan et al., 2006; Valente et al., 2011) the major antifungals in nutmeg EO have not been identified. Such studies are important as they

may lead to new antifungals that would be particularly interesting in the case of *A. flavus* and *A. ochraceus*, potentially dangerous and hard to manage.

We have previously reported that the crude nutmeg EO inhibited the radial growth of *Colletotrichum gloeosporoides*, *C. musa*, *Fusarium oxysporum*, *F. semitectum, A. niger* and *A. glaucus* (Valente et al., 2011). This activity led us to extend our study to *A. flavus* and *A. ochraceus* as well as to identify which of the twenty-eight compound(s) in the crude nutmeg were responsible for the activity. Hence, in this study, we report a) the antifungal activities of the crude and purified nutmeg-EOs against *A. flavus* and *A. ochraceus*; b) identify the major antifungals in the purified EO by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) and c) quantify (relative %) the major antifungals in the crude nutmeg EO by gas chromatography (GC) with a flame ionization detector (FID).

2. Materials and Methods

2.1 Solvents and TLC Analysis

The solvents used for extraction and purification were ACS grade (Vetec, Rio de Janeiro) and HPLC grade (Sigma Aldrich) for chromatographic analysis. All solvents were distilled before use. Thin layer chromatography (TLC) analyses were conducted on silica gel plates and preparative TLC plates (20 × 20 cm, 1-mm thick, 60 GF_{254}, Merck, Rio de Janeiro, RJ, Brazil).

2.2 Plant Material and Hydro-Distillation

Nutmeg seeds, grown in Bahia, Brazil were purchased from a local supermarket. Sixty g of the seeds were ground in a blender (3 L capacity) with 1.2 L of distilled water and the resulting mixture was distilled in a 3 L round bottom flask for 3 h. About 800 mL of the distillate were collected and extracted twice with 150 mL of dichloromethane. The combined extracts were dried with anhydrous sodium sulfate; dichloromethane was evaporated in a rotatory evaporator at 30 ºC under reduced pressure (20 mm Hg) and the residue (EO) was weighed and redissolved in hexane to obtain a concentration of 1 g/mL.

2.3 Antifungal Activity

Antifungal activity of the crude EO was tested on potato-dextrose agar (PDA) using poison food assay PFA (Dhingra & Sinclair, 1995) at concentrations of 0.1 and 0.3%. To obtain a uniform dispersion, the EO was dissolved in methanol (1:1, v/v), and the mixture was added to a molten culture medium. Ten mL of the medium were poured into 9-cm culture plates. The blank was run in exactly the same way without the EO. The medium in each plate was spot-seeded with the conidia of either of the test fungus. Colony diameter was measured on the 6^{th} day after incubation at 25 ºC. All tests were performed in triplicate. The percent growth inhibition was calculated according to the equation: Inhibition (%) = 100 – [(100*B)/A] where A is the control and B is the radial growth in the treatments.

2.4 Separation of the Major Antifungals by Preparative TLC-Bioautography

Preparative TLC-bioautography (Wedge & Nagle, 2000) was conducted with dichlorometane: hexane (8:2) as the elution solvent. An aliquot of the hexane extract containing 100 mg of the EO was placed on each of the 9-TLC plates and developed in the eluting solvent. After TLC the solvent was evaporated at room temperature, 50 mL of molten PDA containing 100 mg/L streptomycin sulfate and conidia of the fungi (10^3/ml) were spread on each TLC plate and placed in a sterilized tray humidified with cotton soaked in water. The tray was covered with a plastic film and incubated for 7 days at 25 ºC. The blank was prepared in exactly the same way without the EO. The region of the TLC plate without fungal growth, considered to be the active fraction, was delineated, scrapped and extracted twice by shaking for two hours with 200 mL of dichloromethane. The mixture was filtered, dehydrated with anhydrous sodium sulfate, and the dichloromethane was evaporated in a rotary evaporator to obtain a residue that was weighed and re-dissolved in hexane to obtain a concentration of 1g/mL and purified by semi-preparative normal phase high-performance liquid chromatography (NP-HPLC).

2.5 Purification of the Major Antifungals by Semi-Preparative NP-HPLC

A fraction of the hexane extract containing 60 mg was injected on a chromatograph with a Si column (250 × 21 mm, 5 μm) and a Si pre-column (50 × 21 mm, 5 μm) (both purchased from Supelco, Bellefonte, PA, USA) with isocratic elution with dichloromethane: hexane (9:1, v/v) at a flow rate of 6 mL/min. The effluent was monitored at 280 nm. Two major fractions were collected and their purities confirmed by analytical NP-HPLC and GC-MS analysis.

Analytical NP-HPLC was carried out on a Si column (250 × 46 mm, 5 μm) and a Si-pre-column (50 × 46 mm, 5 μm) (Supelco, Bellefonte, PA, USA) with isocratic elution with dichloromethane: hexane (9:1, v/v) at a flow rate

of 1 mL/min. GC-MS data were obtained on a gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan, model QP 5000 and software program-Classs-5000, Version 1.2) fitted with an auto sampler, workstation and a database (Wiley 229) with about 350,000 entries. Fused capillary column (30 m x 0.25 mm; film thickness of 0.25 µm) coated with the DB-5 stationary phase was purchased from Supelco (Bellefonte, PA). The GC oven temperature was programmed from 60 ºC (1 min hold) to 240 ºC (9 min hold) at the 3 ºC /min heating rate. One microliter of the sample, dissolved in hexane (10%), was injected by the split mode (1:20) with the split vent being closed for 30 sec. Helium was the carrier gas at flow rate of 1.33 mL/min. The mass spectrometer was scanned from *m/z* 40 to 350 in the electron impact mode (70 eV).

2.6 Identification of the Major Antifungals by NMR and GC-MS

The two analytically pure compounds isolated with semi-preparative NP-HPLC were identified by NMR and GC-MS. One and two-dimensional (hetero nuclear multiple bond connectivity-HMBC and hetero nuclear quantum connectivity HMQC with field gradient) NMR (^{1}H and ^{13}C) were recorded on a Varian Inova spectrometer equipped with indirect detection probe, operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C, using chloroform-d as solvent. The chemical shifts are reported in ppm relative to TMS.

GC-MS identification was carried out as previously described, based on Similarity Index provided by the built-in database of the system and standards.

After identification of the two compounds by NMR and GC-MS, the relative % in the crude nutmeg EO was carried out by GC, using a Shimadzu gas chromatograph (Kyoto, Japan, model GC 17A) with an auto injector, a workstation, and a flame ionization detector. Fused capillary column (30 m × 0.25 mm by 0.25 µm) coated with the DB-5 stationary phase were purchased from Supelco (Bellefonte, PA). Oven temperature was programmed from 60 ºC (1 min hold) to 240 ºC (9 min hold) at 3 ºC /min with the injector and detectors at 220 and 250 ºC, respectively. One µl of the crude EO dissolved in hexane (10% in hexane) was injected by the split mode (1:5) with the split vent being closed for 30 seconds with nitrogen as the carrier gas at a flow rate of 1.33 mL/min.

3. Results and Discussion

3.1 EO Yield

The hydrodistillation of the nutmeg seeds produced a colorless/pale yellow EO with a characteristic odor. The EO yield was 7.1%, based on dry weight basis, which was very similar to 6.9%, reported by Spricigo et al. (1999). Yields ranging 5 to 15% were reported by Somani et al. (2008). The amount of EO found in the seed is known to vary with plant origin, soil and climate, etc (Pino & Borges, 1999).

3.2 Antifungal Activity of the Crude EO, Identification and Quantification of the Major Antifungals of the EO

At a concentration of 0.1% the EO inhibited *A. flavus* and *A. ochraceus* growth by 43 and 65%, respectively. At a concentration of 0.3%, *A. flavus* and *A. ochraceus* inhibitions were 84 and 79%, respectively. (Table 1). Chatterjee (1990) investigated 12 essential oils, including nutmeg for prevention of fungal growth/infection of *A. flavus*, *Curvularia pallescens, Chaetomium indicum, A. glaucus, A. niger* and *A. sydowi* in stored maize grains. As expected, the response of growth/infection varied with the oil. The most effective were cassia, clove and geranium EOs with EFLs (effective levels) of 30 µg/g of the grain. Nutmeg EO, on the other hand was much less effective with an EFL of 50 µg/g of the grain. Varying fungal activity was recorded with the EOs derived from 20 spices against 9 fungi, including nutmeg, *A. niger*. While some EOs inhibited all test fungi, response of other EOs, like the nutmeg varied with the fungal species (Kamble & Patil, 2008). The inhibitory effects of essential oils againt *alternaria alternata* by five EOs (thyme, sage, nutmeg, eucaptus, and cassia) were studied (Feng & Zheng, 2007). Out of the five EOs studied, cassia oil and thyme oil were the most effective at 300-500 ppm provoking 100 and 62.0% inhibitions, respectively. At the same concentration, nutmeg did not produce any inhibition. Atta-ur-Rahman et al. (2000) prepared EO from 7 plants and investigated their antifungal activity against *A. flavus, A. niger, Candida albicans, F. oxysporum, Microsporum canis, Pseudallescheria boydii, Trichophyton mentagrophytes and T. simii*. Poor activity was reported against *A. glaucus* and *A. flavus* with nutmeg EO. We have reported a wide antifungal spectrum of nutmeg EO (Valente et al., 2011). The crude EO at a concentration of 0.1% inhibited radial growth of *C. gloeosporoides* (98%), *C. musa* (97%), *F. oxysporum* (75%), *F. semitectum* (78%), *A. niger* (71%), and *A. glaucus* (60%). Growth inhibition increased from 85% to 100% at a concentration of 0.3%.

It must be pointed out that all literature studies, except one (Atta-ur-Rahman et al., 2000) on antifungal properties of the nutmeg EOs were conducted with EOs obtained from commercial sources, without knowledge of their freshness or chemical composition. EO freshness is important as it is well known that the chemical composition of the EO changes during storage (Sanford & Heinz, 1971). Both the chemical composition and

freshness are intimately related to its antifungal activity and, hence, it is important that antifungal data must be obtained with fresh EO. Apparently, inconsistent antifungal activities of the EO reported in the literature could be due to different chemical compositions along with other factors, such as methodologies, concentrations of EO, age of the EO used, etc. Although Atta-ur-Rahman et al. (2000) used EO extracted by them for antifungal activities, the poor antifungal response against *A.* flavus and *A. ochraceus* could probably be related to the inherent chemical composition of the EO and the methodology (Suhr & Nielsen, 2003),

A. flavus and *A. ochraceus*, potentially hazardous to human health and animal health, are hard to manage. Hence, new antifungals are of great interest. Despite the fact that some studies on antifungal activity of crude nutmeg EO have been conducted, the actual antifungals have not been narrowed down.

We have previously reported antifungal activity of crude nutmeg EO against six storage fungi and have also characterized twenty eight compounds by GC-MS and standards (Valente et al., 2011). In this study, it was possible to identify the major antifungals in nutmeg EO, quickly using TLC-bioautography, which, despite offering a great potential, is relatively little used (Wedge & Nagle, 2000). In this technique, a crude EO is separated by conventional TLC and then sprayed by a fungal suspension of interest. "Clear zones" on the sprayed TLC plates suggest the presence of antifungals that are scrapped from the TLC plate, solvent extracted, purified and identified.

Nutmeg EO after preparative TLC-bioautography presented one active band from which two compounds were identified by NMR and GC-MS. Myristicin or 4-methoxy-6-(prop-2-enyl)benzo[*d*][1,3]dioxole was identified by NMR (Tables 2 and 3) and the following mass spectral data: *m/z* (%): 193 (11), 192 (M^+, 100), 191 (11), 161 (21), 133 (19), 131 (25), 119 (29), 103 (15), 91 (42), 79 (18), 77 (28), 65 (33), 53 (20), 39 (35). Safrole or 1-(prop-2-enyl)benzo[*d*][1,3]dioxole was the second compound identified based on NMR (Tables 4 and 5) and the following mass spectral data: *m/z* (%): 163 (9), 162 (M^+, 100), 161 (27), 135 (33), 131 (54), 104 (53), 91 (8), 78 (28), 77 (51), 51 (46), 39 (28). Safrole and myristicin were identified and confirmed through standards isolated. The relative % of myristicin and safrole in the crude and EO was 10.8 and 2.9%, respectively, being within the range reported in the literature. The following values of myristicin were reported in the literature: 0.2-14.6% (Sanford & Heinz, 1971); 3.8% (Forrest & Heacock, 1972); 14% (Shenk & Lamparsky, 1981); 9.73% (Archer, 1988); 0.5-13.5% (Pino & Borges, 1999); 16.2% (Jukić, et al., 2006). Safrole values reported in the literature were 0.3-4.6% (Sanford & Heinz, 1971); 1.9% (Forrest & Heacock, 1972); 3.3% (Shenk & Lamparsky, 1981); 2.46% (Currò et al., 1987); 2.16% (Archer, 1988); 0.1-3.2% (Pino & Borges, 1999); 3.9% (Jukić, et al., 2006). Both the isolated EO fraction, myristicin isolated and standard , myristicin presented similar activity (Table 1).

Table 1. Percent radial growth inhibited* of *Aspergillus flavus* and *A. ochraceus* by crude nutmeg essential oil (EO), the active fraction (AF), myristicin (My_I) isolated by semi-preparative TLC-bioautography and standard myristicin (My_S) at concentrations of 0.1 and 0.3% after 6-day incubation at 25 ºC

Fungus evaluated	Radial growth inhibited (%) by							
	EO (concentration %)		Active fraction (concentration %)		My_I (concentration %)		My_S (concentration %)	
	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3
A. flavus	43.1± 3.2	84.4± 1.2	44.2± 3.2	89± 1.9	45.0± 3.2	91± 2.9	45.9± 1.2	85± 1.7
A. ochraceus	65.2± 4.2	79.4± 1.9	65± 1.2	80± 4.2	68.1± 4.1	82.9± 2.6	68.2± 4.2	80± 1.2

* Average of three determination.

Table 2. ^{1}H NMR ($CDCl_3$, 500MHz) data for myristicin or 4-methoxy-6-(prop-2-enyl)benzo[*d*][1,3]dioxole

δ (chemical shifts) ppm	Assignment
3.32	dt, 2H, *J* = 6.8, 1.6 Hz, HMBC C-1', C-5', C-3, H-1
3.92	3H, s, HMBC C4', OCH_3),
5.10 and 5.11	1H, dqua, *J* = 10.0, 1.6, 17.0, 1.6 Hz, HMBC C-1, H-3
5.94	1H, ddt, *J* = 17.0, 10.0, 6.8 Hz, HMBC C-6', H-2
5.96	2H, s, OCH_2O), 6.38 (1H, d, *J* = 1.2 Hz, HMBC C-1, C-1', C3', H-5'
6.42	1H, d, *J* = 1.2 Hz, HMBC C-1, C-3', C-5', H-1'

Table 3. ^{13}C NMR ($CDCl_3$, 125MHz) data for myristicin or 4-methoxy-6-(prop-2-enyl)benzo[*d*][1,3]dioxole

δ (chemical shifts) ppm	Assignment
40	CH_2, C-1
56.7	OCH_3, C-4'-OCH_3
101.5	CH_2, OCH_2O
102.8	CH, C-1'
107.8	CH, C-5',
116.2	CH_2, C-3,
133.7	qC, C-3',
134.8	qC, C-6'
137.6	CH, C-2
143.7	qC, C-4',
149.0	qC, C-2'

Table 4. $1^{1}H$ NMR ($CDCl_3$, 500MHz) data for safrole or 1-(prop-2-enyl)benzo[*d*][1,3]dioxole

δ (chemical shifts) ppm	Assignment
3.30	dt, 2H, *J* = 6.8, 1.7 Hz, HMBC C-1', C-5', C-3, H-1
5.05 and, 5.06	each 1H, dqua, , *J* = 10.1, 1.7, 17.0, 1.7 Hz, HMBC C-1, H-3
5.92	2H, s, OCH_2O
6.63	5.93 (1H, ddt, *J* = 17.0, 10.1, 6.8 Hz, HMBC C-6', H-2
6.63	1H, dd, *J* = 7.8, 1.5 Hz, HMBC C-1, C-1', C3', H-5'
6.68	1H, d, *J* = 1.5 Hz, HMBC C-1, C-3', C-5', H-1'
6.74	1H, d, *J* = 7.8 Hz, HMBC C-2', C-6', H-4'

Table 5. ^{13}C NMR ($CDCl_3$, 125MHz) data for safrole or 1-(prop-2-enyl)benzo[*d*][1,3]dioxole

δ (chemical shifts) ppm	Assignment
40.1	CH_2, C-1
101.1	CH_2, OCH_2O
108.4	CH, C-4'
109.3	CH, C-1',
115.9	CH_2, C-3,
121.5	CH, C-5',
134.0	qC, C-6'
137.7	CH, C-2,
146.3	qC, C-3),
147.9	qC, C-2'.

4. Conclusions

Myristicin was responsible for the EO activity against *A. flavus* and *A. ochraceus*. Although safrol was also identified as a minor component in the active fraction of the crude nutmeg EO, its effect on the antifungal activity was not determined and thus its contribution to the antifungal activity is unknown. The role of safrol in the antifungal activity on *A. flavus* and *A. ochraceus* should be investigated. In addition, effects of myristicin and safrol, alone and together, on other fungi should be further studied. Myristicin and safrol could serve as lead compounds to develop other fungicides. Preparative TLC-bioautography should be more extensively studied to determine active compounds in EOs.

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